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Joint Toxicity of Cadmium and Phenanthrene in the Freshwater Amphipod *Hyalella azteca*

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Abstract. The joint toxicity of combined metals and polynuclear aromatic hydrocarbons is poorly understood and may deviate from the summed concentration responses of the individual pollutants. The freshwater amphipod *Hyalella azteca* was exposed to sediment-amended Cd and phenanthrene (Phen) individually and in combination using United States Environmental Protection Agency 10-day sediment toxicity bioassays with lethality and growth end points. The lethal joint toxicity of Cd and Phen was investigated separately in 24-, 48-, and 72-hour aqueous exposures. In sediment exposures, a sublethal concentration of Phen (144 mg kg^{-1}) in combination with Cd increased mortality across a range of Cd concentrations and decreased the 10-day LC_{50} for Cd from 523 mg kg^{-1} (461 to 588, 95% confidence interval [CI]) to 263 mg kg^{-1} (214 to 312, 95% CI). In contrast, sublethal concentrations of Phen had no effect on the lethal toxicity of Cd in aqueous exposures. Combined sediment-amended Cd and Phen acted independently on growth rate. Rate decreases were driven primarily by Cd. Our findings indicated that association with sediment influences the joint toxicity of Cd and Phen. Thus, mixtures of Cd and Phen may cause synergistic or independent toxicity in *H. azteca* depending on the end point investigated and the experimental protocol employed. As an implication of these results, the interpretation of standardized toxicity bioassays, including whole-effluent toxicity tests and single-compound toxicity tests, must be made with caution. These assessment protocols may underestimate potentially hazardous mixture effects in sediment environments. Therefore, risk assessment protocols for environments containing metal–PAH mixtures must include robust methods that can detect possible interactive effects among contaminants to optimize environmental protection.

Mixtures of heavy metals and polynuclear aromatic hydrocarbons (PAHs) are becoming increasingly prevalent in benthic and wetland sediments as a result of urbanization and industrial contamination (Sanger *et al*. 1999a, 1999b). The joint toxicity of these contaminants to organisms in natural environments can be complex and is related to the chemistries of the individual compounds, environment-specific bioavailability, toxicologic modes of action, and possible pharmacologic interactions among contaminants once bioaccumulated (Cassee *et al*. 1998; Broderius 1991). Current hypotheses regarding chemicals with ''dissimilar'' toxicology (*i.e.*, metals and hydrocarbons) suggest that their joint toxicity is independent (Price *et al*. 2002; Broderius 1991). However, published studies that have investigated the joint toxicity of metals and PAHs have indicated that toxicity may be concentration additive, independent, synergistic, or antagonistic (Millward *et al*. 2004; van den Hurk *et al*. 1998b; Babu *et al*. 2001; Moreau *et al*. 1999; Gust and Fleeger, in press). These responses have also been observed to vary among species (Lemaire-Gony and Lemaire 1992; George and Young 1986), among toxicologic end points (Lemaire-Gony *et al*. 1995; Gust and Fleeger, in press), and within end points (Moreau *et al*. 1999; van den Hurk *et al*. 1998a).

The purpose of the present study was to examine the joint toxicity of metal–PAH mixtures in *H. azteca* using cadmium (Cd) and phenanthrene (Phen) as model contaminants in both sediment- and aqueous-toxicity bioassays. *Hyalella azteca* is a toxicant-sensitive epibenthic freshwater amphipod routinely used in standard testing procedures designed to assess contaminant toxicity and sediment quality (United States Environmental Protection Agency [USEPA] 2000; American Society for Testing and Materials 2003). The efficacy of these bioassays for assessing impact in combined metal–PAH exposures was investigated. It has been suggested that metals bioaccumulate and exert toxicity in *H. azteca* primarily through exposure to the overlying water associated with contaminated sediment (Borgmann *et al*. 2001; Borgmann and Norwood 1999; Warren *et al*. 1998). As well, equilibrium partitioning theory indicates that the dissolved fraction of nonionic organic chemicals (*i.e.*, PAHs) in sediment porewater is responsible for contaminant bioaccumulation and toxicity in aquatic organisms (Di Toro *et al*. 1991). Although the contribution of dissolved metal and PAH to toxicity have each been individually well demonstrated, studies including those of Ingersoll *et al*. (2000) and Wang and Fisher (1999) indicated that benthic invertebrates' exposure to sediment-associ-

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ated contaminants can influence bioaccumulation and toxicity. Furthermore, the combined effects of these contaminants in sediment exposures are unknown, and it is unclear if the combined effects are equivalent in sediment and aqueous exposures. We hypothesized that Cd and Phen would have independent joint toxic effects in *H. azteca* and that there would be no difference in *H. azteca*'s response to combined Cd and Phen when comparing sediment and aqueous exposures. Potential causative mechanisms explaining deviations from the previously mentioned hypotheses are discussed and posed for future investigation.

Materials and Methods

Laboratory Culture and Test Organisms

Hyalella azteca (Saussure) cultures were initiated in fall 2002 and were maintained in 20-L aquaria maintained at 23° ± 1°C. Aquaria were filled with dechlorinated tap water of which >50% volume was siphoned off and replaced 3 times/wk. Aquaria were individually aerated and experienced a photoperiod of 12 hours of indirect light to 12 hours of darkness. Amphipods were fed dried, senesced maple leaves, which were added as needed.

Test Methods

All containers and apparatus used to conduct both sediment and aqueous experiments were acid cleaned before use. Sediment toxicity bioassays for Cd and Phen were conducted based on USEPA method 100.1 for *H. azteca* 10-day survival and growth (USEPA 2000). Ten randomly selected *H. azteca* were exposed in 400-ml plastic beakers containing 100 ml test sediment and 200 ml overlying dechlorinated tap water. Static-renewal water-replacement procedures were conducted in which the entire water volume was replaced daily. All beakers were maintained at 23° ± 1°C with a photoperiod of 16 hours of light to 8 hours of darkness. The amphipods were acclimated to this photoperiod for 1 day before initiation of the experiment. Each sediment treatment consisted of 5 replicates, and treatment replicates were arranged and maintained in a completely randomized design. Subadult *H. azteca* (2 to 3 weeks old) were collected by sieving through 1000- and 500-μm mesh stacked sieves, and individuals retained on the 500-μm sieve were used in the toxicity bioassays (Driscoll *et al*. 1997b). This age class is recognized as being as sensitive to Cd as 1–2-week-old animals, and their larger size increases ease of recovery and improves the accuracy of mortality determinations (USEPA 2000). During bioassays, animals were fed 1.5-ml aliquots of yeast, Cerophyl, and trout chow (USEPA 2000) daily. Observation of exposure chambers during bioassays suggested daily meals were ingested completely.

Dissolved oxygen concentration and the temperature in overlying water were monitored daily in three randomly selected replicates using an Orion model 820 oxygen meter (Orion Research, Boston, MA). Overlying water pH and ammonia concentrations were measured from five randomly selected replicates at experiment initiation and termination. pH was measured with a Fisher Accumet model 805MP pH meter (Fisher Scientific, Pittsburgh, PA), and ammonia concentrations were monitored using an NH_3/NH_4 aquarium test kit (Aquarium Pharmaceuticals, Chalfont, PA).

At the experiment's end, amphipods were collected by washing the contents of each beaker on a 250-μm sieve. Sieve contents were rinsed into a counting dish, and the number of living and dead amphipods was determined. Missing amphipods were considered to be dead, and percent mortality was calculated. Growth rate was quantified by subtracting the mean dry weight of 5 groups of 10 randomly selected individuals collected at experiment initiation from the dry weight standardized by the number of surviving animals for each replicate at the end of the 10-day bioassay.

Aqueous lethality bioassays for Cd and Phen were conducted using 10 randomly selected *H. azteca* inserted into 100-ml glass beakers containing 80 ml treatment solution in 4 replicates. Glass beakers were used to minimize Phen binding to exposure chambers. Cadmium and Phen were dissolved in dechlorinated tap water to create treatment solutions. Phenanthrene was first dissolved in a small volume of acetone carrier and then added to water. The acetone concentration in water was 0.1 ml l^{-1}, and a procedural control was conducted to test for acetone effects. Static-renewal water replacement was conducted by replacing the entire water volume twice daily. Test solutions were prepared no more than 1 hour before water renewal. Cadmium concentrations in water-only exposures have been demonstrated to remain stable between static renewals, whereas Phen concentrations may vary by nearly 50% (Gust and Fleeger 2005). Therefore, only initial Cd concentrations were measured, and aqueous Phen concentrations were measured before and after each renewal. Water-quality parameters were measured as previously described.

Sediment Preparation

Sediment was collected from Bayou Manchac, a rural freshwater bayou near Baton Rouge, LA, that has had no history of industrial activity. Levels of trace and heavy metals (Gust, unpublished) and PAHs (Lu, personal communication, October 2004) detected in Bayou Manchac sediment suggest only background concentrations. Sediment was sieved through a 1-cm screen and frozen and thawed twice to eliminate native macroinvertebrates. Sediment was homogenized and stored in the dark at 4°C. Total organic carbon content of the sediment was determined to be 2.59% ± 0.23% using a Perkin Elmer 2400 CHN Series II elemental analyzer (Norwalk, CT). Samples were refluxed for 6 hours in concentrated HCl to eliminate inorganic carbonate and then oven dried at 70°C before analysis. The wet-to-dry ratio of the saturated sediment was 2.2:1.

Phenanthrene (98% purity; Aldrich Chemical, Milwaukee, WI) was amended to sediment by dissolving the chemical in high-performance liquid chromatography (HPLC)–grade hexane and then volatilizing the solvent in a high-purity nitrogen gas stream to coat the inside walls of opaque glass jars. The appropriate mass of wet sediment to achieve a targeted concentration was calculated using the sediment wet-to-dry ratio, then that mass was added to each jar and tumbled on a roller mill at room temperature (23° ± 1°C) for 28 days. Phenanthrene concentrations were measured using HPLC. Two replicates of each Phen-amended sediment treatment were frozen, freeze dried, and homogenized, and preweighed quantities were transferred to a glass extraction vessel. Sixty ml 1:1 mixture of HPLC-grade acetone and hexane was added to the dried sediments. The solvent–sediment combination was sonicated for 20 minutes and then allowed 24 hours for solvent extraction of Phen. After extraction, the solution was decreased in volume by 90% by way of volatilization in high-purity nitrogen gas stream and then brought up to the initial volume with acetonitrile. Samples were analyzed using a Hewlett-Packard 1100 series (Hewlett-Packard, Palo Alto, CA) high-performance liquid chromatograph. Both aqueous Phen and sediment–Phen extract concentrations were determined by reverse-phase HPLC employing an HC-ODS Sil-X, 5-μm particle diameter packed column. Phen was monitored by ultraviolet detection. Five minutes of isocratic elution with acetonitrile/water (4:6) (v/v) were followed with linear gradient elution to 100% acetonitrile during 25 minutes at a flow rate of 0.5 ml/min (USEPA 1986). Two-way analysis of variance (ANOVA) comparing measured

sediment–Phen concentrations at day 0 and day 10 suggested that Phen concentrations were equivalent ($p > 0.05$).

Cadmium chloride (98% purity; Sigma Chemical, St. Louis, MO) was dissolved in deionized water and then slowly amended to a specific mass of wet sediment (determined by wet-to-dry ratio of sediment) to achieve targeted sediment concentrations. Each Cd solution was added drop-wise to sediment in plastic jars undergoing mixing with a hand-held kitchen mixer. For joint toxicity bioassays, Phen was amended to sediment (as previously described) before addition of Cd. Day 0 sediment Cd concentrations were measured using inductively coupled argon plasma spectrophotometry. Two replicates of each Cd treatment were freeze dried, milled, and weighed. Cadmium was extracted by refluxing the sediments in 5 ml hot trace metal–grade HNO_3 for 24 hours. The resulting solution was then decreased in volume to 1 ml by way of volatilization and then diluted to 50 ml with deionized water. Samples were mixed and then left to sit overnight to allow sediment to settle. Both aqueous Cd and sediment–Cd extracts were analyzed using a Jarrell-Ash Model 855 ICP-AES.

Sediment Toxicity Bioassays

Hyalella azteca were exposed to sediment-amended Cd at concentrations of 2, 220, 437, 646, 819, 1050, and 1400 mg kg^{-1} (measured concentrations). Mortality data were analyzed using probit analysis to generate 10-day LC_{50} values with 95% confidence intervals (CIs) using SAS software (release 8.2; SAS, Cary, NC). The effect of Cd on *H. azteca* growth rate was tested using one-way ANOVA calculated with SigmaStat 2.01 software (Jandel Scientific, San Rafael, CA).

Hyalella azteca was exposed to sediment-amended Phen at concentrations that did not exceed sediment saturation as derived by equilibrium partitioning theory (approximately 450 mg kg^{-1}). Phenanthrene exposure concentrations were 0, 32, 58, 116, 225, and 333 mg kg^{-1} (measured concentrations). The mean mortality across sediment–Phen treatments was only 5% ± 8%; therefore, an LC_{50} for Phen could not be determined. Linear regression was used to test the relationship between Phen and mortality, and one-way ANOVA was used to test for Phen effects on growth rate.

An experiment using a randomized design and 2 × 6 factorial treatment arrangement was used to test for joint toxic interactions between Cd and Phen in sediment exposures. The design included a 0 mg kg^{-1} Phen treatment combined with 2, 216, 397, 613, 821, and 1034 mg kg^{-1} Cd and a treatment containing 144 mg kg^{-1} Phen combined with 2, 213, 275, 651, 861, and 1068 mg kg^{-1} Cd (all concentrations represent measured values). Ten-day LC_{50} values with 95% CIs were calculated. Because of complete mortality in the upper range of Cd-alone treatments, growth rate effects for the combined contaminants were tested using two-way ANOVA on only the first four treatments comprising a 2 × 4 treatment arrangement. Water-quality measurements were collected in the Cd-alone and Cd–Phen mixture treatments and compared using Student *t* test (or Mann-Whitney test for data sets with nonnormal distributions).

Aqueous Joint-Toxicity Bioassay

An experiment incorporating a randomized design with 3 × 5 factorial treatment arrangement was used to test for joint toxic interactions between Cd and Phen in aqueous exposures. Phenanthrene treatments included 0- and 150-μg L^{-1} treatments (nominal) and an acetone carrier control. Range-finding experiments indicated that 150 μg L^{-1} Phen (nominal) caused no mortality in *H. azteca*. Phenanthrene concentration in the 150-μg L^{-1} Phen (nominal) treatment was variable and ranged from an average of 119 ± 10 μg L^{-1} on fresh renewal and then decreased to 39 ± 11 μg L^{-1} before renewal. Phenanthrene treatments were combined with 0.0, 2.7, 5.4, 11.7, and 22.7 μg L^{-1} Cd (measured concentrations). Cadmium concentration dynamics have been observed to be less pronounced as evidenced by losses of <15% between renewals (Gust and Fleeger 2005). Mortality counts were taken at 24, 48, and 72 hours, and LC_{50} values with 95% CIs were calculated. Water-quality measurements were collected for Cd alone and Cd–Phen mixture treatments and compared using Kruskal-Wallis ANOVA.

Nonoverlapping 95% CIs were used as criteria for identifying significant differences in mortality among concentration response curves. Effects of Cd and Phen on growth rate were tested using two-way analysis of covariance with SAS software, and a significant interaction term was used as criteria for interactive toxicity between the contaminants.

Results

Water-Quality Measures

No measured water-quality parameter deviated from standards established by the USEPA (2000) in any experiment conducted. In sediment bioassays, only dissolved oxygen differed significantly among Cd-alone versus Cd–Phen mixture treatments ($p = 0.034$). The mean dissolved oxygen concentration for the 10-day bioassay was 6.14 ± 0.41 mg L^{-1} for the Cd alone treatment and 5.92 ± 0.27 mg L^{-1} for the Cd-plus-Phen treatment. No statistically significant differences among treatments ($p > 0.05$) were detected for any of the water-quality parameters measured in the aqueous toxicity test.

Cd and Phenanthrene Individual Toxicities in Sediment Exposures

The resulting LC_{50} value for *H. azteca* exposed to sediment-amended Cd was 484 (417 to 550) mg kg^{-1} (95% CI in parentheses; Fig. 1A). Cadmium significantly affected growth rate (Fig. 1B; one-way ANOVA, $p < 0.001$). Cadmium concentration of 2437 mg kg^{-1} caused a significant decrease in growth rate compared with the control ($p < 0.05$).

There was no relationship between Phen and mortality ($p = 0.530$, $R^2 = 0.0142$; Fig. 1C), and mortality among all treatments was low. The highest mean mortality was observed in the control (12% ± 8%), and mean mortality across all treatments was 5% ± 8%. Phenanthrene significantly decreased *H. azteca* growth rate ($p = 0.007$); however, there was no clear relationship between Phen concentration and the decrease in growth rate (Fig. 1D). Dunnet's multiple comparisons test ($p = 0.05$) indicated significant decreases in growth rate compared with the control and the intermediate Phen treatments (57, 116, and 225 mg kg^{-1}) but not at the lowest or highest Phen concentrations (32 and 333 mg kg^{-1}, respectively).

Cd and Phenanthrene Joint Toxicity in Sediment Exposures

Although Phen did not elicit mortality, Phen increased the lethal toxicity of co-occurring Cd in sediment exposures

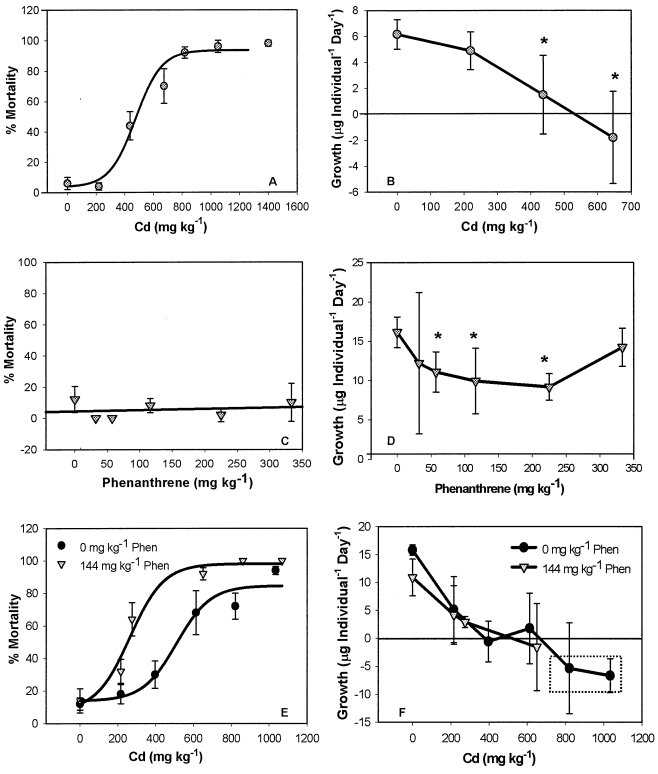

Fig. 1. The effect of sediment-amended Cd on survivorship (**A**) and growth rate (**B**); the effect of sediment-amended phenanthrene on survivorship (**C**) and growth rate (**D**); and the effect of combined sediment-amended Cd and Phen on survivorship (**E**) and growth rate (**F**) in *H. azteca* during 10-day exposures. The points represent means ± 1 SD (n = 5). In the single-contaminant growth plots, (*) signify statistically significant differences ($p < 0.05$) from control as derived by Dunnett's test. In panel (F), to maintain an orthogonal data set for statistical analysis, data points within the dashed box were not used in statistical analysis (see Materials and Methods section). Cd and phenanthrene concentrations are expressed in mg kg^{-1} dry weight sediment.

(Fig. 1E). In the joint toxicity sediment bioassay, the LC_{50} for sediment-amended Cd alone was 523 mg kg^{-1} (461 to 588, 95% CI), which is similar to the LC_{50} determined in the independent bioassay listed previously. In contrast, the LC_{50} determined for Cd combined with 144 mg kg^{-1} Phen was 263 mg kg^{-1} (214 to 312, 95% CI).

The growth rate concentration–response for combinations of sediment-associated Cd and Phen did not differ significantly from the concentration response generated for Cd alone (Fig. 1F). Results of ANCOVA on ranked data indicated that Cd significantly decreased *H. azteca* growth rate ($p < 0.0001$) but that Phen did not ($p = 0.0781$). No significant interaction was found among the contaminant treatments ($p = 0.2482$).

Cd and Phenanthrene Joint Toxicity in Aqueous Exposures

In the aqueous bioassay, Phen did not significantly increase the lethal toxicity of Cd. The concentration-response curves (not shown) and corresponding LC_{50} values and 95% CIs (Table 1) were generally equivalent among the 0-µg L^{-1} Phen, 150-µg L^{-1} Phen, and acetone carrier control treatments for each time period. The only detectable difference among treatments involved a decrease in Cd toxicity when combined with 150 µg L^{-1} Phen at the 48-hour time period (Table 1).

Discussion

Cadmium–Phen mixtures in sediment exposures caused synergistic lethal toxicity in *H. azteca* (Fig. 1E). The 10-day LC_{50} for Cd in sediment was decreased by nearly 50% from 523 (461 to 588, 95% CI) to 263 mg kg^{-1} (214 to 312, 95% CI) when combined with a sublethal concentration (144 mg kg^{-1}) of Phen. Conversely, in aqueous exposures there was a predominantly independent effect of Phen on Cd lethal toxicity. Although there was synergistic lethality associated with Cd–Phen mixtures in sediment, the pollutants caused an independent effect on *H. azteca* growth.

Toxicity in Single-Compound Exposures

The concentration of Cd required to elicit mortality in *H. azteca* was as much as five orders of magnitude higher in sediment than in dechlorinated tap water. These results are consistent with those found by Borgman *et al.* (1991). Decreased metal toxicity in the presence of sediments as been well documented (Ankley 1994; Borgman *et al.* 1991). The toxicity of many metals is directly associated with the total amount of noncomplexed metal found in the dissolved phase (Di Toro *et al.* 2001). Cadmium body burdens in amphipods have been shown to reach equilibrium within 10 days (Clason and Zauke 2000). Based on this observation, it was presumed in the present study that *H. azteca* exposed to Cd alone and the Cd–Phen mixtures approached equilibrium tissue Cd concentrations.

Cadmium significantly decreased *H. azteca* growth rate in sediment exposures. Decreased *H. azteca* growth rate has previously been shown to be a sensitive indicator of Cd contamination in sediments (Milani *et al.* 2003). In the present study, there was no difference in end-point sensitivity to Cd when comparing lethality and growth rate. Neither end point indicated a significant difference compared with the control below a Cd concentration of approximately 400 mg kg^{-1}.

Sediment-amended Phen did not elicit mortality even when concentrations approached equilibrium with the sediment organic carbon fraction (333 mg kg^{-1}). Similarly, >90% survival occurred in *H. azteca* exposed to 1270 nmol g^{-1} (256 mg kg^{-1}) fluoranthene in natural sediment exposures lasting 10 and 16 days (Driscoll *et al.* 1997a). In the aqueous exposures conducted in the present study, Phen ranged from 5% to 10% of saturation and did not elicit mortality. Lower-molecular-weight PAHs such as fluoranthene, anthracene, and Phen are bioaccumulated quickly in *H. azteca* and reach tissue equilibrium in as little as 1 day from either sediment or aqueous exposures (Driscoll *et al.* 1997a, 1997b; Landrum and Scavia 1983). Therefore, in the present study it was reasonable to assume that *H. azteca* approached steady-state Phen body

Table 1. Aqueous Cd LC_{50} values (μg L^{-1}) and corresponding 95% CIs for Cd alone, Cd including acetone carrier, and Cd including phenanthrene (Phen) treatments for *H. azteca*.

	24 h LC_{50}	95% CI	48 h LC_{50}	95% CI	72 h LC_{50}	95% CI
Cd alone	10.0	(8.8–11.6)	3.5*	(2.9–4.2)	1.9	(1.4–2.4)
Cd and acetone	10.4	(8.9–12.2)	4.1	(3.2–5.1)	2.0	(1.5–2.5)
Cd and Phen	12.6	(8.1–20.5)	5.3*	(4.4–6.4)	2.5	(1.9–3.1)

The target Phen concentration in the Cd and Phen treatment was 150 μg L^{-1}. LC_{50} values are given for 24, 48, and 72 h exposures. Values including asterisk (*) represent nonoverlapping 95% CIs within a given exposure time.

concentrations during both sediment and aqueous bioassays. The tendency of *H. azteca* to quickly reach steady-state PAH body concentrations of relatively low magnitude may be related to its ability to efficiently metabolize and eliminate PAHs (Driscoll *et al*. 1997a, 1997b; Landrum and Scavia, 1983). Thus, *H. azteca*'s ability to regulate total PAH body burden may have contributed to the high survivorship observed in the present Phen bioassays.

Phen significantly decreased growth rate, although there did not appear to be a direct negative relationship between Phen concentration and growth. Growth rate was significantly decreased in Phen treatment concentrations >58 through 225 mg kg^{-1} but not at the highest treatment of 333 mg kg^{-1}. Although growth rate decrease was a more sensitive indicator of sediment–Phen contamination than lethality, our results indicated that growth rate responses to Phen were inconsistent.

Synergistic Toxicity in Hyalella azteca

Cadmium has been found to impair PAH metabolism in the estuarine fish *Fundulus heteroclitus* by inhibiting hepatic cytochrome P450 (Cyp1A) enzyme activity (van den Hurk *et al*. 1998a), and synergistic lethal joint toxicity was associated with this response (van den Hurk *et al*. 1998b). Cytochrome P450 pathways allow organisms to biotransform, detoxify, and excrete an array of organic xenobiotics including PAHs (Klaassen 2001). In the present study, although Phen caused no mortality alone and appeared to increase the toxicity of Cd in Cd–Phen sediment mixtures, the synergistic toxicity observed in *H. azteca* may have been the result of Cd-mediated interference with Phen detoxification rendering Phen toxic.

George and Young (1986) showed that the PAH 3-methylcholanthrene delayed the onset of metallothionein induction in plaice *Pleuronectes platessa* hepatocytes by 6 days compared with plaice exposed to Cd alone. Metallothioneins are evolutionarily widespread, low-molecular-weight proteins that bind various metals *in situ*, thus significantly decreasing toxicity (Roesijadi 1992). Therefore, Phen-mediated inhibition of metallothionein induction in *H. azteca* could have increased lethality in *H. azteca* exposed to the Cd–Phen mixture.

In sediments, biotic exposure to metals may be altered by PAH. Millward *et al*. (2004) found increased sediment retention of Cu and Cr in flow-through microcosms when diesel fuel was a co-contaminant. The differential retention–flux and/or bioavailability of contaminants caused by co-contaminants may alter exposure to and hence the overall toxicity to organisms in sediment environments. Little research has been conducted examining the effect of PAH on the bioavailability of metals. However, results in related research employing sediment bioassays identical to those used in this study (Gust and Fleeger 2005) indicated that Phen does not alter Cd concentrations found in the dissolved phase (the most bioavailable metal fraction). The results of Gust and Fleeger (2005) also indicate that the observed increase in *H. azteca* mortality, when exposed to Cd–Phen mixtures in sediment, is independent of the Cd concentration dissolved in overlying water.

Although the bioavailability of Cd may not be altered by Phen, modifications in animal behavior or physiology may alter metal exposure and bioaccumulation (Gust 2005). Behavioral or physiological responses of *H. azteca* to sediment-associated Phen may have increased Cd bioaccumulation and overall toxicity by way of mechanisms that are not manifested in aqueous exposures. For example, Fair and Sick (1983) observed that the Black Sea bass (*Centropristis striata*) assimilated higher concentrations of Cd in various tissues from food containing Cd and naphthalene compared with food containing Cd alone. Recently conducted experiments with *H. azteca* indicated that Phen increased the Cd bioaccumulation rate in sediment exposures but not in water-only exposures (Gust and Fleeger 2005). In the present study, the observed increase in *H. azteca* mortality in sediment exposures, but not in aqueous exposures, may have resulted from Phen-mediated increases in Cd bioaccumulation rate.

It has been suggested that metals bioaccumulate and exert toxicity in *H. azteca* primarily from exposure to overlying water associated with contaminated sediment (Borgmann *et al*. 2001; Borgman and Norwood 1999; Warren *et al*. 1998). However, the critical body residue of Cd has been shown to be 2- to 2.6-fold higher when accumulated from sediment rather than from aqueous sources (Borgmann *et al*. 1991). This indicates that sediments may provide an alternative source for Cd uptake that may not reach the site of toxic action. Deposit-feeding organisms have been shown to bioaccumulate the majority of their metal body burden through ingestion of metal-contaminated sediment (Wang and Fisher 1999). Furthermore, it seems logical that ingestion of metal-contaminated sediment could lead to differential exposure among tissues compared with uptake from aqueous sources. These observations indicate that synergistic toxicity between Cd and Phen may occur if Phen enhances the partitioning of Cd to the site of toxic action or if Phen renders Cd toxic to tissues typically unassociated with Cd toxicity. Autoradiography with radioactive Cd would allow visualization of the location of metals to determine if sediment exposure alters the distribution of Cd among tissues (Rouleau *et al*. 2001).

Concentration-dependent interactions in which synergisms and antagonisms occur at different exposure concentrations of metals and PAH have been documented (Moreau *et al*. 1999; van den Hurk *et al*. 1998b) and may potentially explain the differences in results between sediment and aqueous expo-

sures. In both the sediment and aqueous toxicity bioassays in the present study, Cd concentrations ranged from those causing low to complete mortality, and all Phen concentrations were sublethal. Based on the equilibrium-partitioning coefficient for Phen in the test sediment, the dissolved Phen concentration found in porewater of the sediment bioassay was comparable with that used in the aqueous bioassay within a factor of 2. Therefore, exposure concentrations used in both the sediment and aqueous tests were similar, thus indicating that the differential toxicity found between these bioassays is most likely not the result of a concentration-dependent interaction between Cd and Phen.

Although synergistic lethal effects were found between Cd and Phen in sediment bioassays, the effect of the combined contaminants was independent for growth rate. This observation brings into question the choice of appropriate experimental end points when assessing environmental risk. Sublethal end points, including growth rate, may be considered more sensitive indicators of sediment toxicity than lethality. This study indicated that growth rate was not more sensitive than lethality for Cd or Phen toxicity. Additionally, growth rate was not indicative of the deleterious synergistic lethal toxicity found between these contaminants. Growth rate of *H. azteca* may not be an appropriate end point in acute toxicity bioassays.

Implications

Based on current hypotheses in chemical mixture toxicology, dissimilar contaminants are expected to have independent joint toxicity (Price et *al*. 2002; Broderius 1991). Metals and PAHs represent different chemical classes that exhibit dissimilar toxicology and typically have unrelated environmental chemistries. The results of the present study showed that the assumption of independent toxicity between these chemical classes cannot be made with confidence. Additionally, the results of this study, as well as examples from the literature, indicated that joint toxic interactions between metals and PAH are possible if not probable. These observations call into question the accuracy of risk-assessment protocols employing independent or additive toxicity as their underlying assumptions for metal–PAH-contaminated environments. As a broader implication of these results, chemical toxicity protocols should include testing of combined contaminants when the joint toxicity of chemical classes in a given mixture is unknown.

The joint toxic effects of metals and PAHs are potentially species specific (Lemaire-Gony and Lemaire 1992; George and Young 1986), and as demonstrated by the present study, metal–PAH toxicity may be end point specific and dependent on the environmental exposure media. As an implication of these results, the interpretation of standardized toxicity bioassays used to assess environmental quality (including whole-effluent toxicity and single-compound toxicity tests) must be made with caution. For example, increased mortality was observed in *H. azteca* exposed directly to field-collected sediment containing a mixture of metals, PAHs, and polychlorinated biphenyls compared with those exposed only to the overlying water (Ingersoll *et al*. 2000). This demonstrates that the assessment protocols mentioned previously may underestimate potentially hazardous contaminant mixture effects representative of complex sediment environments. Improved understanding of the environment-specific mechanisms influencing interactive toxicity between metals and PAH is required for development of a more predictive global model of metal–PAH toxicity.

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